Effects of Bone Marrow Mesenchymal Stem Cell to Transforming Grow Factor-β3 and Matrix Metalloproteinase-9 Expression in Burns

by Gusti Revilla1

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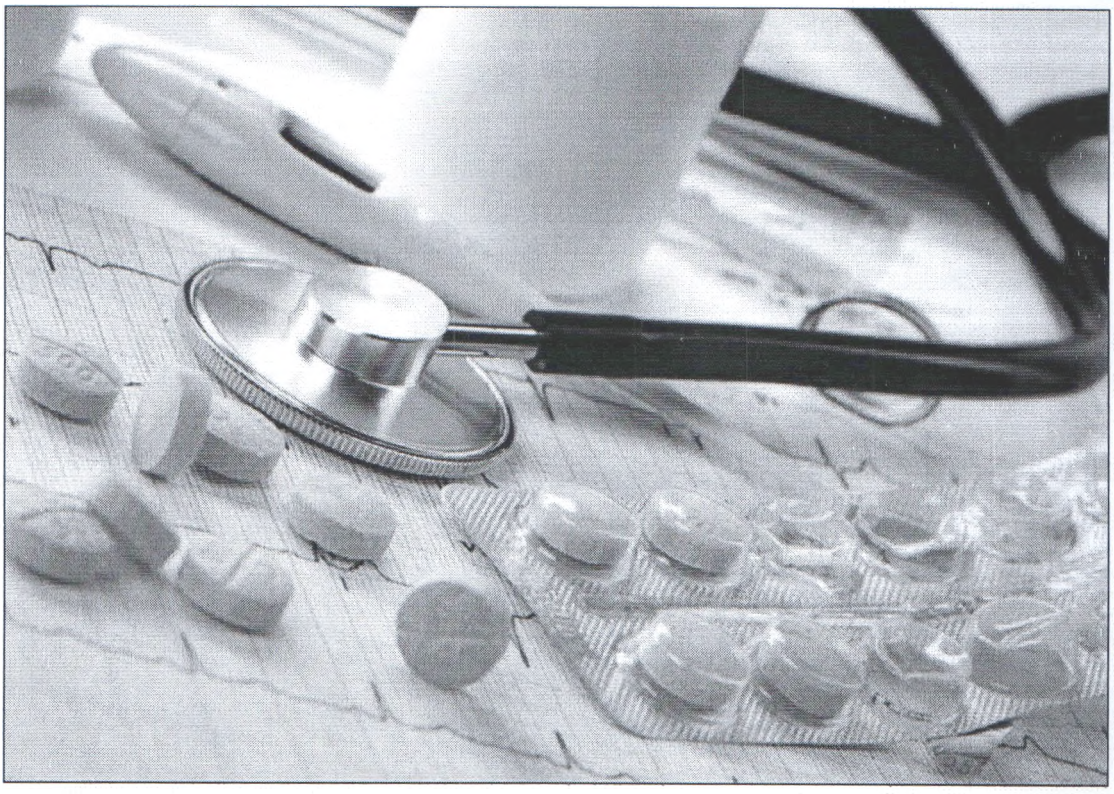

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Research Article

Effects of Bone Marrow Mesenchymal Stem Cell to Transforming Grow Factor-β3 and Matrix Metalloproteinase-9 Expression in Burns

[1]Gusti Revilla, [2]Nita Afriani and [1]Dewi Rusnita

[1]Department of Anatomy, Faculty of Medicine, Andalas University, Jl. Perintis Kemerdekaan, No. 94, P.O. Box 49, 25129, Padang, Indonesia
[2]Department of Histology, Faculty of Medicine, Andalas University, Jl. Perintis Kemerdekaan, No. 94, P.O. Box 49, 25129, Padang, Indonesia

Abstract

Background and Objective: Burn wound is a serious issue in health because of the outcome results the physical disability, influencing the morbidity of the patient, high cost of burn wound treatment. Treatment of full depth burn has been done in various ways by injecting growth factors and skin grafts, but the result has not provided satisfactory, it's due to the formation of scar tissue, the absence of hair follicles and sebaceous tissue. Bone marrow stem cells (BM-MSCs) are strong potential cell in skin tissue regeneration. Study on BM-MSCs administration to burn wound is unclear and this study was performed to identify the formation of granulation tissue and to investigate the expression level of transforming growth factor beta 3 (TGF-β3) and matrix metalloproteinase-9 expression (MM-9). The TGF-β3 plays a role in reducing scar tissue formation and MMP9 serves as collagen destruction. **Materials and Methods:** Ten Wistar rats were divided into two groups, i.e., control group was treated with PBS and treated group was injected with BM-MSCs. A burn wound was made on dorsal area of rat. On day 14 after treatment, burn wound was investigated; immunohistochemical examination was performed to identify granulation tissue formation, TGF-β3 and MMP-9 expression. **Results:** The administration of BM-MSCs on day 14 accelerated the burns healing in rat and increased the expression level of TGF-β3 (7.32) and MMP-9 (6.26) significantly, compared with in control group (2.64 and 3.98). **Conclusion:** The BM-MSCs accelerated the burns healing in rat by elevating the expression level of TGF-β3 and MMP-9 which acts in proliferation phase in wound healing.

Key words: Bone marrow stem cells, transforming growth factor beta 3, matrix metalloproteinase-9, burns, wound healing

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Corresponding Author: Gusti Revilla, Department of Anatomy, Faculty of Medicine, Andalas University, Jl. Perintis Kemerdekaan, No. 94, P.O. Box 49, 25129, Padang, Indonesia Fax: +6275132838

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The BM-MSCs is a multipotent progenitor stem cell, differentiated into chondrocytes, osteoblasts, adipocytes, myocytes, fibroblasts, epithelial cell myofibroblasts, endothelial cells and neurons[1,2]. The BM-MSCs have strong potential in skin tissue regeneration[3,4], but study on the administration of BM-MSCs for skin wounds is effective and unclear[5].

Previous studies have been performed on wound incisions[4,6,7] and burns[8,9]. The BM-MSCs showed to accelerate wound closure with good healing quality, so BM-MSCs are indispensable in skin wound repair[10]. The BM-MSCs on angiogenesis demonstrated to increase capillary density and related with the formation of new blood vessels. The formation of new blood vessels is affected by the ability of BM-MSCs to differentiate into endothelial cells, by releasing the soluble factors[11], increasing migration, proliferation and fibroblast genes to increase wound strength[12], blood vessels formation in smooth muscle that play a role in integrating endothelium of the vessel wall[13]. The BM-MSCs also increase endothelial cell proliferation and vascular permeability[14].

Endothelial cell proliferation and the formation of new blood vessels are affected by soluble factors such as vascular endothelial growth factor (VEGF), angiogenic-1 factor, platelet derivate growth factor (PDGF) and Transforming Growth Factor-Beta 3 (TGF-β3). The TGF-β3 is a growth factor which playing a role to form granulation tissue and reduce scars tissue formation. Previous study found that TGF-β3 in incision wound declined α actin smooth muscle formation and decreased collagen tissue maturation, the condition at the end related to granulation tissue formation[15]. Study by *in vivo* showed that TGF-β3 together with VEGF and PDGF work simultaneously to elevate integrity of capillary to form new blood vessels fast[16].

Angiogenesis occurs in hypoxia condition, causes the elevation of free radicals; reactive oxygen species (ROS) and nitrite oxide (NO). Free radicals increase growth factor and matrix metalloproteinase (MMP). There are several types of MMP, i.e., collagenase (MMP-1, MMP-8 and MMP-13), gelatinose (MMP-2 and MMP-9) and stromelysin (MMP-3, MMP-10, MMP-11)[17]. The MMP 9 is a type of MMP which plays a role during the forming of granulation tissue. Free radicals increase bioactivity and the expression of MMP-9. MMP-9 is synthesized by macrophage and keratinocyte. The MMP-9 in angiogenesis breaks the collagen and protein in lamina basalis of blood vessel and elevates the activity of VEGF. The role of MMP-9 in inflammation and proliferation phase is by supporting the growth and migration of keratinocyte. The increase of MMP re-gulation during re-modeling phase of tissue, together with PDGF, is to break the old collagen and then reduces the forming of scars tissue[18,19]. The TGF-β3 and MMP-9 are together to play role during angiogenesis process in wound healing.

The study was performed to identify the administration of allogenic BM-MSCs in the expression of TGF-β3 and MMP-9 on rat wound healing process.

MATERIALS AND METHODS

The study was performed on September-December, 2016 in Laboratory of Anatomy, Faculty of Medicine, Andalas University, Padang.

Preparation of BM-MSc: The BM-MSCs were obtained from stem cell biobank which commercially was purchased in Stem Cell Laboratory, Institute of Tropical Disease Laboratory, Faculty of Medicine, Airlangga University, Surabaya. The BM-MSCs were placed in a monolayer tube, cells were separated and calculated. The dosage of MSC for each rat was 1 mL 2×10^6 cells mL^{-1}.

Preparation of rat: A total of 10 Wistar rats weighing between 180-200 g was acclimated for a week into the surrounding environment. Each rat was cage individually; water and chow of rat were given *ad libitum*. The lightning and humidity of cage were arranged in such a manner for well-established conditions. Rats did not move out from laboratory during application performed. Rats divided into two groups: Control group (injected with PBS), treated group (injected with BM-MSCs) with five rats in each group and burns were induced on all the rats according to Paramonov and Chebotarev[20]. A small area (1.5×1.5 cm) of full thickness burns was induced on the dorsal area of each rat. The control group received PBS treatment, whereas, another group was treated with BM-MSCs. The study has received approval from the Ethics Committee of the Faculty of Medicine andalas University, Padang.

Procedure: The rats were anesthetized with xylazine and ketamine (ratio 1:1), the dorsal area of rats were shaved. Dorsal side was chosen as this area was not reachable by the rats and therefore; the wound healing was not disturbed. A special metal, 1.5×1.5 cm with holder was heated in 100°C of hot water for 30 min and applied to dorsal area of rat for 20 sec to induced full thickness burn wound. In control group, rat was injected with PBS 1 mL and treatment group was injected with 1 mL BM-MSCs at dose 2×10^6 cells mL^{-1}. The burn wound was

covered by tegaderm film and elastomull haft. Antalgin as an analgetic was injected to rats during observation process. Rats were sacrificed at 14 days, at which times, skin samples including the wound and 4 mm of the surrounding skin were harvested using a 10 mm biopsy punch. For whole skin mount, the entire wound and surrounding skin was placed on plastic (tissue culture dish) with the dermis side down and photographed immediately.

Immunohistochemical examination: Tissue specimen were fixed with paraffin process by dipping the slides into xylol solution three times for 5 min, rehydrated with ethanol absolute and ethanol 70%, respectively for 2 min. Slide was blocked with 3% H_2O_2, incubated with anti-TGF-β3 and MMP-9 (mouse anti rat 1:50) (Gene Tex, USA) for 30 min at room temperature, followed by incubation with a secondary antibody (rabbit anti mouse biotinylated antibody label) for 30 min. Slide was labeled with Streptavidin HRP for 30 min and added chromogen substrate for 3-5 min, counter stained then with Mayer hematoxylin. Image was taken by using Olympus BX 31 400X magnification.

Observation of TGF-β3 and MMP-9 immunohistochemical examination: The determination of TGF-β3 and MMP-9 expression level was calculated based on the number of macrophage which finding in stained tissue. The observations were performed in five different fields of view in microscope. Protein expression of TGF-β3 and MMP-9 was assessed by histochemistry staining using monoclonal antibody. Brown color result of staining in granulation tissue was examined in five large fields of view. The mean of cell which expressed TGF-β3 and MMP-9 was obtained by totalizing the number of cell in every view and divided with number of field of view.

Statistical analysis: All values are expressed as mean. Student's t-test was performed for comparison of data of samples and analysis of variance. A probability p-value <0.05 was considered significant.

RESULTS AND DISCUSSION

The administration of BM-MSCs in burn wound rat accelerated the wound healing compared with control group. Figure 1 showed that burn wound healing in treatment group was faster than control group in day 14.

The healing of burn wound is a complex process by involving several overlapped phases; inflammation, proliferation (i.e., the forming of granulation tissue, re-epithelialization) and remodeling. The three phases of wound healing correlated with the interaction of mediators, cell and matrix extracellular protein[21,22]. Treatment of burn wound received many attentions by the researchers because burns cause physical damage even death, so that in current time, stem cells are used for therapeutic burns. In this study, the administration of BM-MSCs accelerated the wound closure with good healing quality. Therefore, MSCs are needed in skin wounds repair and the growth or appearance of hair follicles covering the wound. In contrast, there was still no visible reduction of burn wound skin of rats in control group.

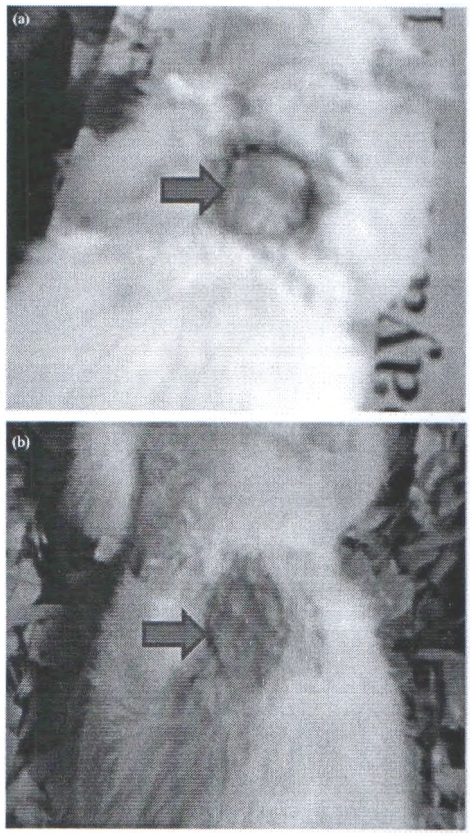

Fig. 1: Condition of burn wound recovery in rat in day 14 (red arrows) (a) Treatment group with BM-MSCs administration and (b) Control group with PBS administration. Burns on the surface of treated rats' skin have dried up and hair begins to grow on the burn area. While in the control group, healing burns look slow and there are parts of the wound that has not dried up

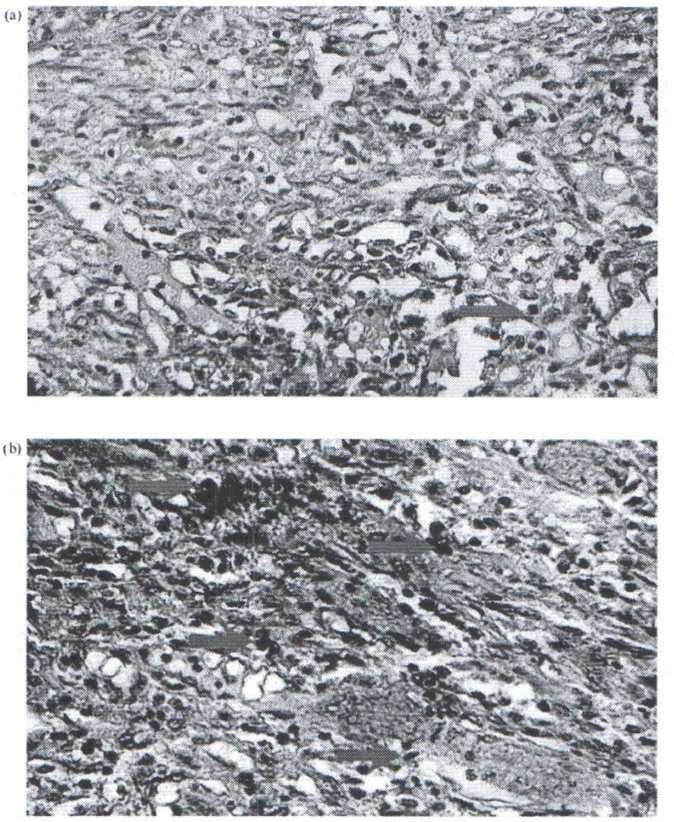

Fig. 2(a-b): Result of staining toward TGF-β3 expression in burns of rat skin tissue in (a) Control group. Red arrow shows positive staining of round cell (b) Treatment group. The stained cells microscopically show the cytoplasm of round cells are browner than control group (400X magnifying). It was due to the increase of TGF-β3 expression level after the administration of BM-MSCs
Red arrows showed positive staining

Previous studies which concern about mesenchymal effect of stem cells on burn wound healing have also been widely practiced. The BM-MSCs accelerated wound healing by reducing infiltration of inflammatory cells, anti-bacterial, speeded up the formation of new blood vessels, the formation of granulation tissue, epithelialization, increased the thickness of type 1 collagen and the formation of skin glands[23-29].

Expression of TGF-β3 and MMP-9: The expression of TGF-β3 and MMP-9 proteins was assessed by immunohistochemical examination. Immunohistochemical result of TGF-β3 showed significant differences in the number of round cells in treatment group compared with control group ($p = 0.05$) (Fig. 2a, 3a).

The number of round cells expressing TGF-β3 in control group was less than the treatment group (2.4 cells/field), which was determined by calculating the number of round cells (result of staining) (Table 1).

Expression of TGF-β3 and MMP-9 on rat burns skin: Staining results of TGF-β3 and MMP-9 showed significant differences in the number of brown spherical cells in the treatment group (BM-MSCs) compared with control group ($p = 0.05$) (Fig. 2b, 3b). The role of TGF-β3 and MMP-9 during wound healing is in the inflammatory and proliferative phase. In the inflammatory phase, TGF-β3 and MMP-9 attract/recruit the inflammatory cells to the wound[30,31], whereas, in the proliferative phase, TGF-β3 and MMP-9 help the angiogenesis process granulation

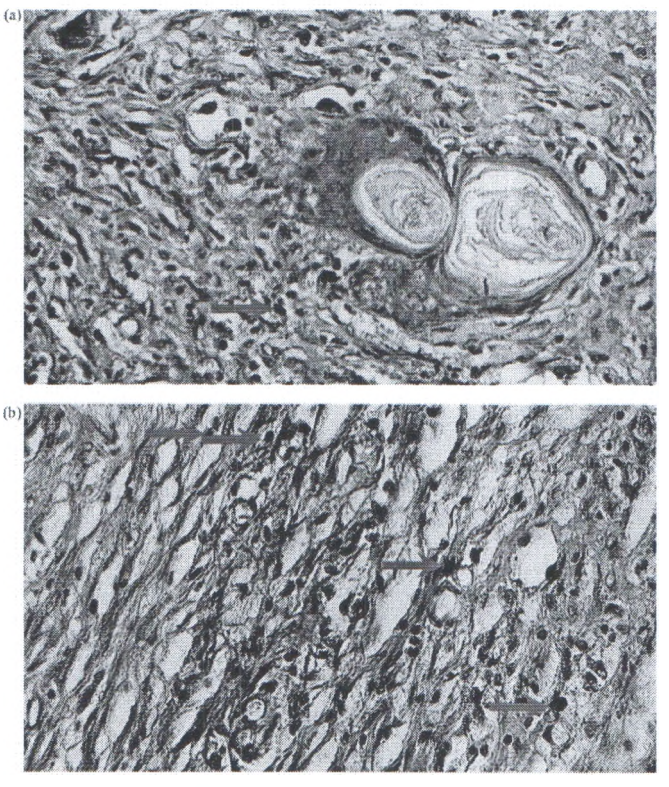

Fig. 3(a-b): Staining result of MMP9 expression in burns of rat skin tissue in (a) control group. Red arrow shows positive staining of round cell (b) Treatment group. The stained cells microscopically show the cytoplasms of round cells are browner than control group (400X magnifying). Red arrows showed positive staining of MMP, browner round cells indicate the increase of MMP level expression after BM-MSCs administration on burns

Table 1: Number of cells expressing TGF-β3 in burn wound after BM-MSCs administration on day 14, performed in five different fields of microscope view

Observation	Control group	Treatment group	p-value
1	3.2 (3-4)	5.2 (4-7)	
2	2.8 (2-3)	6.8 (5-8)	
3	2.0 (1-3)	7.8 (6-9)	
4	2.3 (2-3)	8.3 (7-10)	
5	2.9 (2-4)	8.5 (8-10)	
Total mean	2.64	7.32	0.05*

*Student t-test analysis, p = 0.05, statistically significant

Table 2: Number of cells expressing MMP9 in burn wound after BM-MSCs administration on day 14 in five different fields of microscope view

Observation	Control group	Treatment group	p-value
1	3.3 (2-4)	7.0 (6-8)	
2	3.8 (3-5)	5.3 (5-8)	
3	4.3 (3-5)	8.1 (7-9)	
4	4.4 (4-5)	6.2 (5-7)	
5	4.1 (3-6)	4.7 (4-6)	
Total mean	3.98	6.26	0.05*

*Student t-test analysis, p = 0.05, statistically significant

of tissue, epithelialization and degrading process of EMC. Angiogenesis is a process of new blood vessel capillaries forming, stimulated by several growth factors such as PDGF, VEGF and TGF-β in burn wound area. These growth factors bind to receptors on the surface of endothelial cells in pre-existing venules (parent vessels) and then activate the signals into endothelial cells. The activated endothelial cell releases proteolytic enzymes that dissolve basal membrane of parent vessels. Endothelial cells proliferate or grow out through the basal membrane and migrate to the wound area[32,33].

Paracrine function of BM-MSCs showed that stem cells increase the level of TGF-β3 and MMP-9 secretion. TGF-β3 and

MMP-9 plays a role in proliferation phase to elevate granulation tissue formation and inhibit the deposit of ECM. In remodeling phase of wound recovery, the increase of TGF-β3 expression level inhibits the alteration of fibroblast to myofibroblast, so that the scar formation will be prevent and initiate the organization of collagen (Table 2)[34-36].

The expression of TGF-β3 increased after BM-MSCs administration[37]. Qi *et al.*[38] found that stem cells secrete TNF-a-induced protein 6 (TSG-6) cytokines that act to decrease TGF-β1 and increase TGF-β3 expression levels, thereby the formation of scar tissue in the skin tissues can be suppressed.

The elevation of MMP-9 after BM-MSCs injection gave an impact in increasing of angiogenesis. Jiang *et al.*[39] reported that the administration of BM-MSCs increased the activity of MMP-2 and MMP-9 and then increased the capillary formation of new blood vessels. The growing blood vessels release the matrix metalloproteinase (MMPs) which serves to degrade the surrounding of tissue matrix and synthesize an inter-connected tubular tube to form a vascular loop. The vascular loop then differentiates into the arteries and veins and finally the blood flows into wound area and returns to normal condition[40].

CONCLUSION

This study concluded that stem cell BM-MSCs administration in rats on day 14 accelerated the burn wound healing, increased the expression of TGF-β3 and MMP-9 protein expression. It was due to these two proteins play a role in the formation of new blood vessels and reduces the formation of scars tissue.

SIGNIFICANCE STATEMENT

Study on BM-MSCs administration to burn wound is unclear and this study was performed to investigate the expression level of TGF-β3 and MMP-9 expression. Previous studies have been performed on wound incisions and burns, but study on the administration of BM-MSCs for skin wounds is effective and unclear. This study found that BM-MSCs accelerated the formation of granulation tissue and the TGF-β3 and MMP-9 level was synthesized in balanced expression.

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